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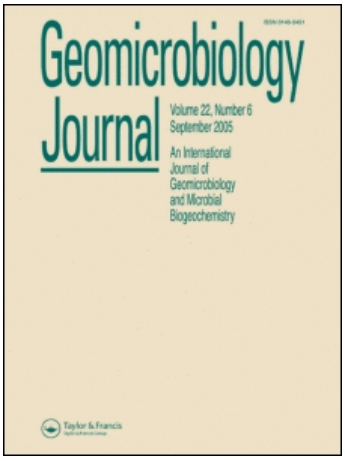

Geomicrobiology Journal
Publication details, including instructions for authors and subscription information:
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Effect of Competing Electron Acceptors on the Reduction of U(VI) by *Desulfotomaculum reducens*

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Online publication date: 08 June 2010

To cite this Article Junier, Pilar , Suvorova, Elena I. and Bernier-Latmani, Rizlan(2010) 'Effect of Competing Electron Acceptors on the Reduction of U(VI) by *Desulfotomaculum reducens*', Geomicrobiology Journal, 27: 5, 435 — 443
To link to this Article: DOI: 10.1080/01490450903480293
URL: http://dx.doi.org/10.1080/01490450903480293

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Geomicrobiology Journal, 27:435–443, 2010
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ISSN: 0149-0451 print / 1521-0529 online
DOI: 10.1080/01490450903480293

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Effect of Competing Electron Acceptors on the Reduction of U(VI) by *Desulfotomaculum reducens*

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The biological reduction of soluble U(VI) to the less soluble U(IV) has been proposed as a strategy to remediate uranium-contaminated sites. However, the majority of the contaminated sites contain, in addition to U(VI), competing electron acceptors (CEAs) that can either enhance or inhibit U(VI) reduction. *Desulfotomaculum reducens* MI-1 is a sulfate-reducing bacterium able to reduce a variety of electron acceptors including U(VI). We characterized U(VI) reduction by *D. reducens* in the presence of pyruvate and three CEAs: sulfate, nitrate or soluble ferric iron. In the presence of sulfate or ferric iron and U(VI), cell growth was driven by respiration of the CEA. Nitrate was not used as an electron acceptor for growth and vegetative cells grew instead by fermenting pyruvate. Sulfate remaining after sulfate reduction has ceased or the presence of nitrate did not affect U(VI) reduction. However, in the case of sulfate, the addition of H_2 after the depletion of pyruvate greatly enhanced U(VI) reduction. Contrary to sulfate and nitrate, the presence of Fe(II), the product of Fe(III) reduction, abolished U(VI) reduction. The results from this investigation suggest that this microorganism and others with similar characteristics may play a role in U(VI) bioremediation efforts but only after the soluble Fe(II) produced by Fe(III) reduction has been advected away.

Keywords biomineralization, bioremediation, metal reduction, subsurface microbiology, sulfate-reducing bacteria

Received 19 October 2009; accepted 10 November 2009.
This work was funded by the Swiss National Foundation Division III through project 33100A0-112337 and through the US Department of Energy grant number DE-FG02-06ER64227. We thank Brad Tebo and Anna Obraztsova for providing us with the *D. reducens* isolate, Felippe de Alencastro and the IIE Central Environmental Laboratory for their great analytical support, Martin Schroth at ETHZ for access to the IC, Subrahmanyam Challapalli at EPFL for help in measuring H_2, and Daniel Alessi for thermodynamic calculations of the nature of the Fe(II) precipitate. We are especially grateful to Dorothy Parker for her thorough and constructive comments on the manuscript. Support for this project was also provided by BER-ERSD project number SCW0041. Portions of this research were carried out at the Stanford Synchrotron Radiation Laboratory, a national user facility operated by Stanford University on behalf of the U.S. Department of Energy, Office of Basic Energy Sciences.
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INTRODUCTION

U(VI) contamination as a legacy of nuclear fuel and weapons production is a severe problem in many U.S. Department of Energy (DOE) sites and similar locales worldwide. A potential solution for the remediation of this contaminant involves *in situ* immobilization of U by the reduction of the hexavalent form (U(VI)) to the less soluble and less bio-available tetravalent form (U(IV)), which precipitates as the mineral UO_2 (Wall and Krumholz 2006). In general, the strategy considered consists of adding an appropriate electron donor that would stimulate the indigenous microbial community and promote the use of U(VI) as a terminal electron acceptor (Anderson et al. 2003; Wu et al. 2007; Yabusaki et al. 2007; N'Guessan et al. 2008; Madden et al. 2009).

However, the majority of the contaminated sites to be remediated contain, in addition to U(VI), other potential electron acceptors that are either naturally occurring (e.g., Fe(III)) or deposited during the uranium fuel and weapon manufacturing process (e.g., NO_3^-, SO_4^{2-}) (Riley et al. 1992). Several studies have evaluated the effect of competing electron acceptors (CEAs) on U(VI) reduction; the results vary greatly -from inhibition to stimulation- depending on the CEA, the microorganism or microbial community and the speciation of U(VI) (Spear et al. 1999; Finneran et al. 2002; Anderson et al. 2003; Pietzsch and Babel 2003; Elias et al. 2004; Istok et al. 2004).

Species from various bacterial genera are capable of U(VI) reduction, for example: *Shewanella* (Lovley 1991), *Geobacter* (Lovley 1991), *Desulfovibrio* (Lovley and Phillips 1992; Lovley et al. 1993) and *Anaeromyxobacter* (Wu et al. 2006), all of which are Gram-negative proteobacteria. However, field studies of U(VI)-reducing microbial communities increasingly suggest the relevance of Gram-positive bacteria, especially Firmicutes, in environmental settings. Firmicutes appear to be responsible for the immobilization of U(VI) after *in situ* biostimulation with acetate at a site belonging to the U.S. Department of Energy, in Rifle (Colorado, USA) (N'Guessan et al. 2008). Communities from acidic subsurface environments contaminated with U(VI) in Shiprock (New Mexico, USA) were also found to be dominated by Firmicutes (Petrie et al. 2003), and enrichments favoring the growth of fermentative bacteria

from the same site revealed the presence of microorganisms related to *Clostridium* spp., which were capable of reducing U(VI) (Madden et al. 2007).

Desulfotomaculum reducens strain MI-1 is an isolate that could serve as a model for these Gram-positive bacteria. It is a sulfate-reducing, spore-forming bacterium isolated from marine sediments contaminated with Cr(VI) and was the first described sulfate-reducer able to couple growth to the reduction of metals (Cr(VI), Mn(IV), Fe(III)) (Tebo and Obraztsova 1998). Despite its marine origin, this organism has been grown under freshwater conditions in our laboratory suggesting that it could live in the subsurface. *Desulfotomaculum reducens* strain MI-1 is capable of fermenting pyruvate to produce acetate, H_2 and CO_2, which is common for many *Desulfotomaculum* species (Widdel 1992). Cultures of *D. reducens* grown in the presence of uranium reduce the radionuclide only after pyruvate has been depleted and growth has ceased. This behavior was attributed to the involvement of spores, produced in the late exponential phase, in U(VI) reduction by *D. reducens*. Spores reduce U(VI) using H_2 as an electron donor but require the presence of a factor present in the spent medium to carry out this process (Junier et al. 2009).

The effect of competing electron acceptors (Fe(III) and SO_4^{2-}) on U(VI) reduction by strain MI-1 was deemed worth investigating because the bacterium is able to couple growth to the respiration of Fe(III) as well as SO_4^{2-} (Tebo and Obraztsova 1998), and the effect of these CEAs on spore-driven U(VI) reduction was unknown. Nitrate is an additional CEA commonly found in U(VI) contaminated areas (Riley et al. 1992). Prior to this work, nitrate respiration by *D. reducens* had not been tested nor had the effect of NO_3^- on U(VI) reduction by this bacterium. The overall goal of this study was to evaluate the effect of competing electron acceptors, specifically SO_4^{2-}, Fe(III) and NO_3^-, on U(VI) reduction by *D. reducens*.

We found that U(VI) reduction by strain MI-1 was not inhibited by the competing electron acceptors NO_3^- and SO_4^{2-} , but was abolished in the presence of Fe(III) or, rather, the product of its reduction, Fe(II).

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MATERIAL AND METHODS

Cultivation Conditions

Desulfotomaculum reducens strain MI-1 was kindly provided by Anna Obraztsova. Standard anaerobic conditions were used throughout the study (Balch et al. 1979). Widdel low phosphate (WLP) medium, modified from Widdel and Bak (1992), was used for growth experiments. In addition to vitamins and trace minerals, the constituents of WLP were as follows (per liter): NH_4Cl, 0.25 g; $CaCl_2.2H_2O$, 0.1 g; $MgCl_2.6H_2O$, 0.5 g; NaCl, 5 g; KCl, 0.5 g; and KH_2PO_4, 0.03 g adjusted to pH 7.2. The medium was dispensed in 50 mL volumes into 100-mL glass serum bottles and autoclaved.

The following solutions were added from sterile anaerobic stocks (final concentration): yeast extract, 0.05%; $NaHCO_3$, 30 mM; pyruvic acid, 10 mM. Additionally, due to the high salt content of the medium, 20 mM 1,4-piperazinediethane sulfonic acid disodium salt monohydrate (PIPES) was added to maintain pH and prevent U(VI) precipitation. The final pH of the medium was 7.2 ± 0.2. All cultures were grown in a 100% N_2 atmosphere. All chemical compounds were obtained from Sigma-Aldrich unless otherwise indicated.

U(VI) Reduction Experiments in the Presence of CEAs

For reduction assays, 100 μM U(VI) (as uranyl acetate), 5 mM sulfate (as Na_2SO_4), 2 mM nitrate (as $NaNO_3$), or 8 mM iron(III) (as soluble ferric citrate) were added from anaerobic stocks to WLP medium. A sporulated culture initially grown in the presence of 20 mM lactic acid and 20 mM sulfate was used as an inoculum (10% vol/vol of inoculum) for 50 mL of medium containing the relevant electron acceptor(s). Throughout this manuscript, 'sporulated culture' refers to a culture that was allowed to form spores but that still contains a majority of cells. Two controls were included in the experiments: one did not contain cells and the other lacked electron donor. All experiments were carried out in biological triplicates. Cultures were incubated at 37°C and sampled using disposable syringes in the anaerobic chamber. The presence or absence of spores was determined by optical microscopy.

Spore Preparation

Pasteurized spore suspensions referred to herein as 'spore preparations' were obtained by centrifuging a 60-hour pyruvate-grown culture at $8000 \times g$ for 15 min and resuspending it in fresh anaerobic medium containing 20 mM acetate and 1 mM sulfate to induce sporulation as described previously (Junier et al. 2009). To prepare a pasteurized spore suspension, the preparation was pasteurized for 20 minutes at 80°C. The final spore preparations were concentrated 100-fold in anaerobic MilliQ water and yielded a spore suspension containing $\sim 1 \times 10^8$ spores per mL as determined by direct counting with a Petroff-Hauser counting chamber and no visible vegetative cells.

U(VI) Reduction in Spent Medium

Spent fermentative medium was obtained from a pyruvate fermentation culture after the depletion of pyruvate. Cells and spores were removed by centrifugation. The supernatant was filter-sterilized in the anaerobic chamber (5% H_2) and transferred anaerobically to a new sterile container. An H_2-depleted spent medium control was obtained by filter-sterilizing H_2-free sulfate reduction spent medium in an H_2-free (N_2-only) glove box. To test U(VI) reduction in either spent medium, aliquots of 5 ml of spent medium were added to sterile Hungate tubes containing either an atmosphere of 100% hydrogen (26 mM H_2 in the tube) or 100% N_2 and amended with a final

concentration of 100 μM anaerobic U(VI) (as uranyl acetate). Prior to U(VI) addition, some of the tubes were also amended with 10 mM of anaerobic Fe(II)-chloride that partially precipitated as Fe(II)CO_3. Unpasteurized spores were added to the assays and U(VI) concentration was monitored over time.

Analytical Methods

Protein from a centrifugally pelleted culture was extracted by incubation at 95°C for 10 min in 0.1% Triton X-100, 0.1% SDS, 10 mM EDTA and 1 mM Tris-HCl. After a 100-fold dilution, the protein concentration was determined using the Bradford assay (Biorad, Hercules, CA). Uranium was analyzed by kinetic phosphorescence analysis (KPA-11A; Chemcheck Instruments, Richland, WA) after anaerobic filtration (Millipore Millex-GV PVDF 0.2 μm). Nitrate and sulfate were analyzed by ion chromatography (DX-500, Dionex, Sunnyvale CA) in an IonPac AS12A column and a 30 mM bicarbonate eluent after filtration and 20- to 50-fold dilution. HCl-extractable Fe(II) was measured with ferrozine as previously described (Sorensen 1982). Total iron in the samples was measured using a Perkin-Elmer 2000 inductively coupled plasma optical emission spectrometer (ICP-OES) in 1% HNO_3. Organic acids were measured using an ion chromatograph (DX-3000, Dionex, Sunnyvale, CA) with an IonPac AS11-HC column. Elution was carried out using a gradient of 0.5 to 30 mM KOH. Hydrogen was measured using a GC-TCD (Hewlett Packard HP6890-TCD) with a carboxen capillary column and nitrogen as a carrier gas.

RESULTS

Effect of Sulfate on U(VI) Reduction

In a culture grown with pyruvate, sulfate and U(VI), sulfate reduction ceased after 30 hours (Fig. 1A) due to the depletion of pyruvate (data not shown). The stoichiometry of pyruvate and sulfate consumption (consumed pyruvate/sulfate ratio of ~3.9) corresponded to the expected ratio 4:1 for sulfate respiration with pyruvate as an electron donor. Acetate and CO_2 are the products of pyruvate respiration whereas acetate, H_2 and CO_2 are the products of pyruvate fermentation. Thus, pyruvate fermentation did not occur as was evidenced by the absence of H_2 production (Table 1). The use of pyruvate as an electron donor instead of a fermentative substrate is dictated by thermodynamic considerations because the $\Delta G^{0'}$ for sulfate respiration with pyruvate as an electron donor is approximately double that of pyruvate fermentation ($\Delta G^{0'}$ fermentation $= -47.3$ versus $\Delta G^{0'}$ sulfate respiration $= -85.3$) (Thauer et al. 1977).

Limited U(VI) reduction was observed in the presence of sulfate: after 180 hours, only 15 μM U(VI) was reduced (Fig. 1A). By 324 hours, only 37 μM was reduced (data not shown) and, over that time frame, 0.5 μmoles U(VI) were reduced per mg protein, significantly less than in the absence of sulfate (Table 1). No significant chemical reduction of U(VI) by hydrogen sulfide was observed in the abiotic control (data not shown).

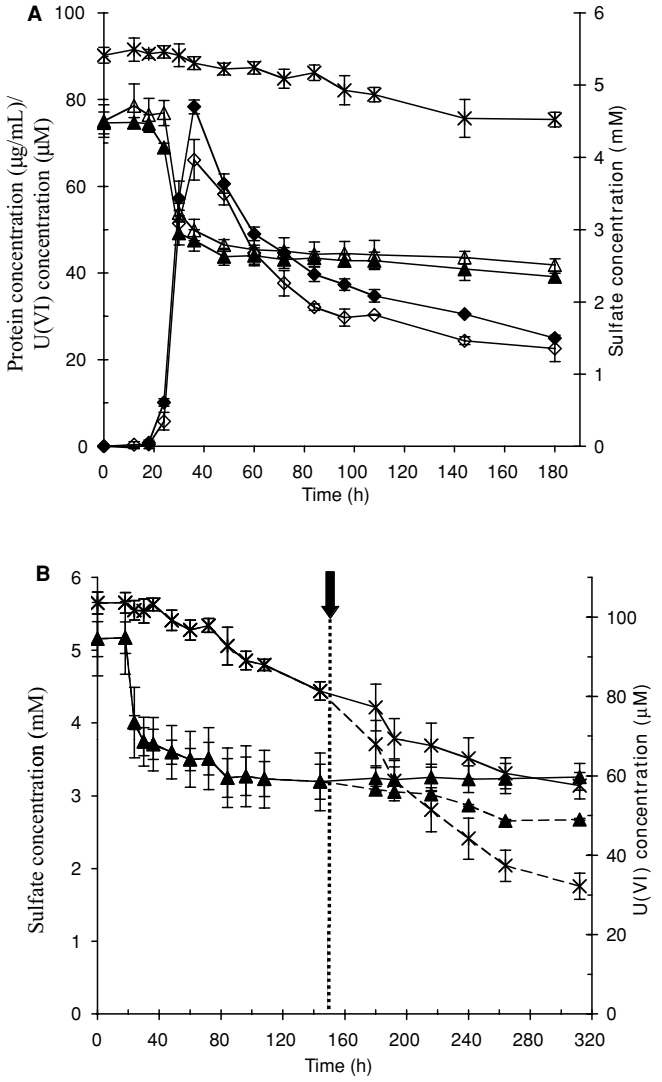

FIG. 1. Sulfate reduction in the presence (filled symbols) and absence (open symbols) of U(VI). (A) Protein (♦ and ◇), sulfate (▲ and △) and U(VI) (∗) concentration. (B) Sulfate reducing culture in the presence of U(VI). Sulfate (▲) and U(VI) (⋆) concentration. After 140 hours (indicated by arrow), H_2 (20 mM) was added in duplicate cultures and the effect on sulfate and U(VI) concentrations observed (dotted lines) as compared to replicate cultures in which no H_2 was added (solid lines). Triplicates were run for all conditions and the error bars represent the standard deviation within the set.

In a previous study, H_2 was shown to be required as an electron donor for U(VI) reduction by spores of *D. reducens* (Junier et al. 2009). Microscopic analysis of the sulfate-reducing culture (Table 2) showed that spores were present at a significant concentration (~12%) in sulfate-reducing cultures (at 108 hours). Thus, we suspected that the absence of H_2 in those cultures could explain the limited U(VI) reduction observed. To test this hypothesis, H_2 was added to a culture after it had stopped reducing sulfate (i.e., after pyruvate was depleted) and U(VI) reduction monitored in the amended as compared to the unamended cultures (Fig. 1B). Addition of H_2 clearly stimulated

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TABLE 1
Maximum biomass (in μg/mL of protein) obtained during the entire growth profile and concentration of H_2 produced (mM) and U(VI) reduced (μmoles per mg protein) after 324 hours of incubation

Condition	Maximum biomass (μg/mL)	H_2 conc (mM)	μmoles U(VI) reduced per mg protein	U(VI) reduced after 324 hours (μM)
Sulfate	66 ± 4.6	0		
Sulfate + U(VI)	78 ± 1.6	0	0.5	37
Nitrate	35 ± 1.5	10 ± 0.3		
Nitrate + U(VI)	36 ± 4.1	9.2 ± 0.8	3.3	119
Fe(III)	70 ± 9.1	1.9 ± 0		
Fe(III) + U(VI)	64 ± 9.2	2.8 ± 0.2	0.0	3
Fermentation*	19 ± 0.2	4.5 ± 0.2		
Fermentation + U(VI)*	18 ± 6	5.4 ± 0.0	4.7	85

Pyruvate was used in all the condtions as the electron donor. * = For comparison the values for fermentation and fermentation + U(VI) are presented in the table.

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U(VI) reduction as compared to the control, indicating that H_2 is an electron donor for U(VI) reduction by *D. reducens*. Sulfate reduction also appears to be stimulated by the addition of H_2, suggesting that H_2 could serve as an electron donor for sulfate reduction by *D. reducens*.

Additionally, spent medium from a sulfate-reducing culture was filter-sterilized and used for U(VI) reduction assays with strain MI-1 pasteurized spores or pasteurized cells. The results show that pasteurized spores can reduce U(VI) when provided with H_2 as an electron donor, despite the presence of 3.2 mM sulfate in the spent medium (Table 3). In the absence of H_2 and in the case of pasteurized cells, there was not significant U(VI) reduction.

Thus, the observed lesser extent of U(VI) reduction in a *D. reducens* sulfate-reducing culture (Fig. 1A) is not directly due to the presence of sulfate but rather to the fact that sulfate respiration does not lead to H_2 production, which is needed for U(VI) reduction. The limited U(VI) reduction catalyzed by sulfate-reducing cultures in the absence of added H_2 is attributed to the injection of small amounts of H_2 from the anaerobic chamber atmosphere (5% H_2) into the culture serum bottles at each sampling event.

TABLE 2
Spore and cell concentrations in various cultures after 108 hours of growth

Growth condition	Cell concentration (cells/ml)	Spore concentration (spores/ml)
Pyruvate fermentation	2.3×10^7	3.2×10^6
Sulfate reduction	4.4×10^7	5.1×10^6
Iron reduction*	1.5×10^7	0

*Spore content was also evaluated in Fe(III)-grown cultures after 200 hours and no spores were detected then as well.

We conclude that sulfate does not act as a direct competing electron acceptor for U(VI) reduction suggesting that spores-driven U(VI) reduction could conceivably take place in the presence of sulfate.

Effect of NO_3^- on U(VI) Reduction

In the case of nitrate as a CEA, we observed little difference in U(VI) reduction in the presence or absence of nitrate during growth of *D. reducens* with pyruvate (Fig. 2 and Table 1). In addition, no detectable nitrate reduction was observed throughout the experiment in the presence or absence of

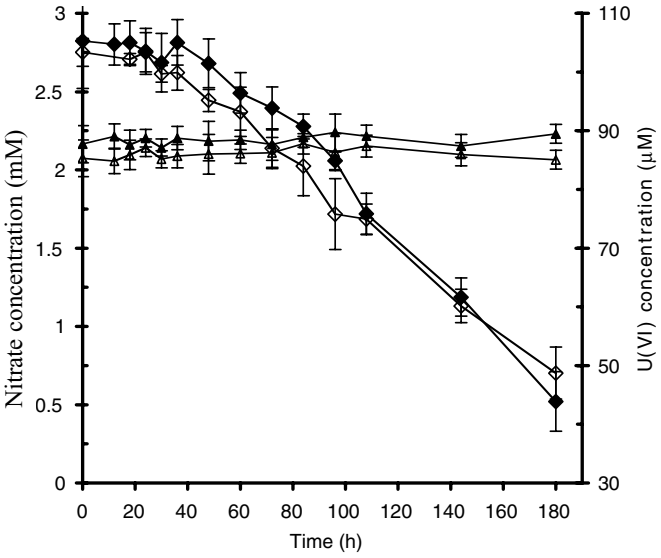

FIG. 2. Nitrate and U(VI) reduction in a growing culture of *D. reducens*. Nitrate reduction in the presence (▲) and absence (△) of U(VI) and U(VI) reduction in the presence (♦) and absence (◇) of nitrate. Triplicates were run for all conditions and the error bars represent the standard deviation within the set.

TABLE 3
U(VI) reduction by spores and cells in the presence of spent pyruvate-free fermentative or sulfate-reducing medium amended with or free of H_2

	% U(VI) reduced		
	Spent fermentative medium	Spent sulfate-reducing medium	
	20 mM H_2 added	20 mM H_2 added	No H_2
Pasteurized spores	73.5 ± 2.4	43.2 ± 4.0	0.5 ± 0.0
Unpasteurized spores	83.9 ± 1.0	ND	2.2 ± 0.1
Pasteurized cells	1.9 ± 5.6	3.2 ± 0.1	1.3 ± 0.0
Abiotic (no spores or cells)	2.1 ± 5.9	0.4 ± 0.1	1.0 ± 0.1

The spore concentration was $1.6 * 10^6$ spores/mL and the cell concentration $2.3 * 10^7$ cells/mL. Spent sulfate-reducing medium contains 3.2 mM sulfate.

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U(VI) (Fig. 2). These results clearly indicate that NO_3^- is not reduced by strain MI-1 and does not act as a competing electron acceptor for U(VI) reduction by strain MI-1 spores.

Effect of Fe(III) on U(VI) Reduction

The reduction of U(VI) was evaluated for cells grown with the competing electron acceptor Fe(III) and pyruvate as an electron donor (Fig. 3). Fe(III) was reduced to Fe(II) and the stoichiometry of consumption of pyruvate and Fe(III) matches the expected stoichiometry for Fe(III) respiration with pyruvate as an electron donor (Fig. 3): 2 moles of Fe(III) per mole of pyruvate. In addition, it is apparent that biomass production is greater when cells are grown with pyruvate and Fe(III) than with pyruvate alone (Table 1), which is consistent with Fe(III) respiration being a more energetic process ($\Delta G^{0'}$ ferric iron respiration = −275.8) (Thauer et al. 1977).

Interestingly, little U(VI) reduction was observed in the entire experiment (Fig. 3A). Even after 324 hours, long after Fe(III) and pyruvate were depleted (data not shown), only 3 μM of U(VI) were reduced despite the presence of H_2 (Table 1). Microscopic observation of Fe(III)-grown cultures showed the complete absence of spores (Table 2) and the association of cells with an Fe precipitate (Fig. 3B). Hence, sporulation was inhibited under these conditions. We suspect that the most likely explanation for sporulation inhibition is the precipitation of an x-ray amorphous Fe(II) carbonate mineral phase. This mineral released gas when acidified and was not identifiable by conventional x-ray diffraction. A thermodynamic model of the possible supersaturated phases under these conditions suggests the formation of a Fe(II) carbonate phase corresponding to siderite (data not shown). The production of $FeCO_3$ was supported by EDX analysis of the Fe(II) precipitates formed after Fe(III) reduction (Fig. 3B). Small area electron diffraction (SAED) also confirmed a poorly crystalline $FeCO_3$ as the main mineral phase (Fig. 3C).

To probe whether the effect of iron extends only to inhibiting sporulation or whether iron may also inhibit U(VI) reduction by spores, a culture grown with Fe(III), U(VI) and pyruvate for 190 hours was amended with spores after pyruvate had been depleted and Fe(III) reduced to Fe(II) (Fig. 4A). This treatment did not initiate U(VI) reduction, even when fermentation spent medium was added. Therefore, U(VI) reduction was inhibited despite the presence of spores, H_2 and spent medium. This result suggests that the presence of Fe(II) not only inhibits sporulation but also interferes directly with U(VI) reduction when *D. reducens* spores are present. However, this inhibition is clearly not solely due to electron acceptor competition between U(VI) and Fe(III) as it persists after all Fe(III) has been converted to Fe(II).

In order to test directly the effect of Fe(II) on U(VI) reduction by spores, anaerobic Fe(II)-chloride was added to fermentation spent growth medium containing H_2 that was later supplemented with an unpasteurized spore preparation (10% vol/vol dilution) and U(VI). We observed partial precipitation of the Fe(II)-chloride (presumably as Fe(II) CO_3) prior to U(VI) addition (data not shown). After U(VI) was added, it remained in solution during the duration of the experiment (Fig. 4B). Spores added to the Fe(II)-treated spent medium did not exhibit U(VI) reduction, while U(VI) reduction took place in the un-treated fermentation spent medium (Fig. 4B). This result suggests that either precipitated and/or soluble Fe(II) inhibited U(VI) reduction by strain MI-1 spores.

DISCUSSION

In this study, we considered the effect of competing electron acceptors on U(VI) reduction by *D. reducens* in the presence of pyruvate as an electron donor. A previous study showed the ability of *D. reducens* to use U(VI) as the sole electron acceptor using butyrate as an electron donor (Tebo and Obraztsova 1998). We found that in the case of pyruvate, growth was preferentially driven by fermentation (pyruvate only or pyruvate and nitrate)

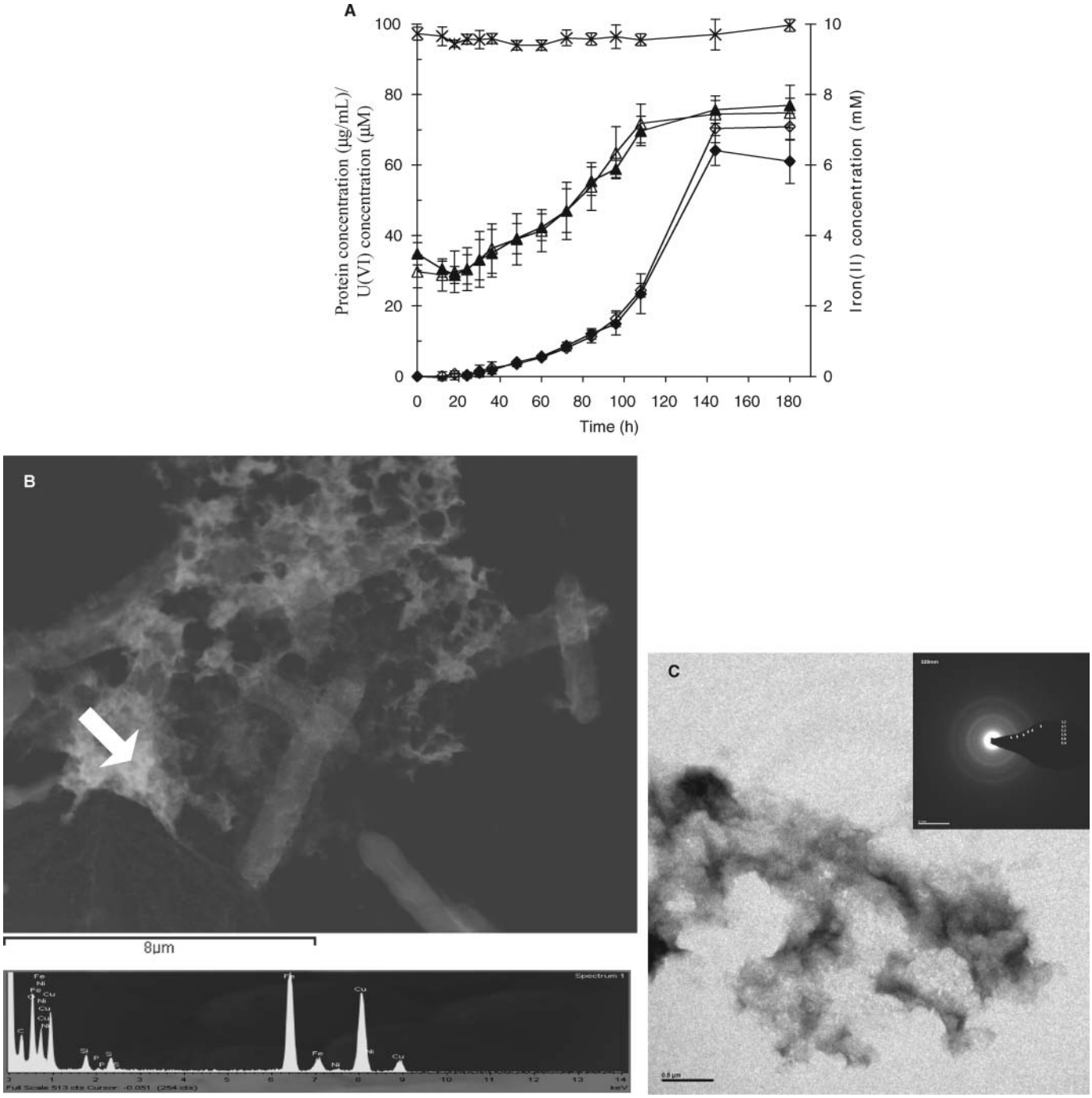

FIG. 3. Fe(III) reduction in the presence (filled symbols) and absence (open symbols) of U(VI). (A) Protein (♦ and ◇), Fe(II) (▲ and △) and U(VI) (∗) concentrations. Triplicates were run for all conditions and the error bars represent the standard deviation within the set. (B) TEM image of Fe(II) precipitates formed after Fe(III) reduction by *D. reducens.* (C) EDX spectra taken in the area indicated by an arrow in B. D. SAED obtained on the precipitates.

or respiration of the competing electron acceptor (sulfate and iron), rather than by U(VI) reduction. The majority of U(VI) reduction occurred after growth ceased and was dependent on the presence of H_2 as an electron donor. It is not straightforward to compare the metabolism of *D. reducens* when butyrate serves as the electron donor to that when pyruvate is present. Thus, it is difficult to comment on the apparent differences between our results and those of Tebo and Obraztsova (1998).

Many U contaminated sites in the United States and in Europe contain high concentrations of nitrate and/or sulfate due to the

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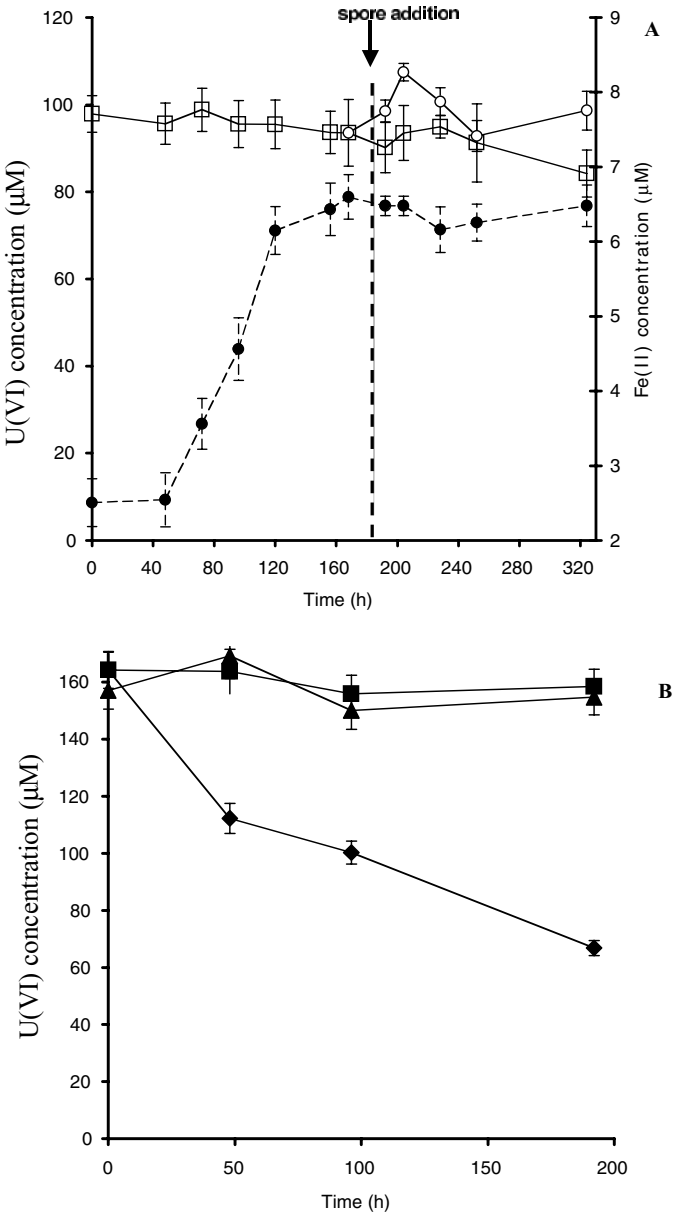

FIG. 4. (A) U(VI) reduction in an Fe(III) reducing culture in which, at 192 hours, the replicates were subjected to separate treatments: amendment with spores (○) and no addition (□). Spent medium was added to one of the two replicate and in all cases there was no effect on U(VI). Fe(II) resulting from Fe(III) reduction is shown with a dotted line (●). Duplicates were run for all conditions and the error bars represent the range. (B) Concentration of U(VI) over time after incubation in fermentative spent growth medium supplemented with spores of *D. reducens* strain MI-1 and H_2 in the presence or absence of Fe(II) chloride. Abiotic control supplemented with 10 mM Fe(II) chloride (■). Spores supplemented with 10 mM Fe(II) chloride (▲). Positive control with spores in the absence of Fe(II) chloride (♦).

use of nitric and/or sulfuric acid for the processing of uranium ore (Riley et al. 1992; Akob et al. 2009). In addition, Fe(III) is naturally occurring in many of the subsurface environments in which U contamination is found (Kelly et al. 2008; N'Guessan et al. 2008). The occurrence of these competing electron acceptors can be problematic for bioremediation efforts because the preferential use of alternative electron acceptors by microorganisms can delay or even completely inhibit U(VI) reduction. In anoxic environments, the range of potential competitive electron acceptors (CEAs) is diverse (e.g., nitrate, manganese, iron and sulfate), and the sequence of use is dictated by availability and redox potential of the acceptors (Lovley 1991; Nealson and Saffarini 1994).

Nitrate is a particularly problematic co-contaminant because it was shown to compete significantly with U(VI) reduction as it is preferentially reduced by *Geobacter* spp. (Istok et al. 2004), microbial consortia (Shelobolina et al. 2003) and acetate-amended aquifer sediments (Finneran et al. 2002). For *D. reducens*, nitrate did not serve as an electron acceptor and U(VI) reduction in the presence of pyruvate appeared to be independent of nitrate (Fig. 2).

Interestingly, recent findings from enrichment cultures in contaminated sediments from the Oak Ridge Field Research Center (OR FRC) also showed almost complete reduction of uranium with little loss of nitrate at slightly acidic pH values (from 5.7–6.2) (Madden et al. 2007). Although *Clostridium*-like bacteria appear to be dominant, the analysis of the microbial community in the enrichments revealed the presence of *Desulfotomaculum*-like microorganisms, particularly at higher pH (pH 6.2) in the presence of methanol and glycerol. The results of the present study suggesting that U(VI) reduction by *Desulfotomaculum* spp. is insensitive to nitrate are consistent with the findings at the OR FRC.

U(VI) reduction in the presence of sulfate has been studied for different SRB (Lovley and Phillips 1992; Tucker et al. 1996; Spear et al. 2000). In many studies, the addition of sulfate to pure SRB cultures or to subsurface sediments stimulated the rate of U(VI) reduction (Abdelouas et al. 2000; Spear et al. 2000). In the case of *D. reducens*, sulfate did not affect U(VI) reduction significantly if the electron donor for U(VI) reduction (H_2) was present.

But U(VI) reduction was severely limited if H_2 was not provided. Sulfate-grown cultures must be amended with the appropriate electron donor, H_2, for U(VI) reduction to occur because pyruvate fermentation and the concomitant release of H_2 are compromised during sulfate respiration. In the presence of H_2, U(VI) reduction proceeded unhindered independently of the excess of sulfate still present in the medium and H_2 was simultaneously used as an electron donor for sulfate reduction (Fig. 1B). Thus, *D. reducens* shows a distinct behavior from other SRB for which sulfate enhances U(VI) reduction (Abdelouas et al. 2000; Spear et al. 2000) in that sulfate does not directly interfere with or promote U(VI) reduction.

Although sulfide has the potential for chemical U(VI) reduction, previous results suggest that, in the presence of a bicarbonate buffered system, sulfide (1 mM) is a poor reductant of U(VI) (Lovley and Phillips 1992; Hua et al. 2006). Similarly, the presence of 2.5 mM sulfide does not induce chemical reduction in the medium used for U(VI) reduction by *D. reducens*, which

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contained 30 mM bicarbonate and 20 mM PIPES as buffers (data not shown).

In the case of Fe(III), another respirable and potentially competing electron acceptor, U(VI) reduction by *D. reducens* was completely inhibited (Fig. 3). Although the absence of spores in Fe(III)-grown cultures could partially explain the lack of U(VI) reduction, the addition of spores did not rescue the activity (Fig. 4B and Table 2). Additionally, an inhibitory effect of soluble and/or solid phase Fe(II) was observed (Fig. 4B). Previous work has shown that Fe(II) accumulation at the surface of *Shewanella* spp. inhibits the cells' reducing activity (Urrutia et al. 1998). Thus, a similar mechanism could be responsible for the Fe(II)-mediated inhibitory effect on U(VI) reduction. An alternative explanation could be that the formation of the Fe(II) precipitate leads to the sorption of a factor present in spent medium, thus effectively removing it from solution. This factor was shown to be a necessary component for spore-driven U(VI) reduction (Junier et al. 2009). The detailed mechanism of inhibition was not investigated but merits some attention.

The presence of Firmicutes such as the genera *Desulfotomaculum* or *Desulfosporosinus* in uranium-contaminated sites has been documented (Chang et al. 2001; Madden et al. 2007; N'Guessan et al. 2008). Based on this work, we expect that microorganisms similar to *D. reducens* would play an important role in the subsurface after Fe(III) reduction is completed, during and after the sulfate reduction phase. In the subsurface, it is likely that Fe(II) would not have a long-lasting deleterious effect on sporulation or U(VI) reduction because precipitation of Fe(II) may not take place and advection will move dissolved Fe(II) away from the area of electron donor amendment over time (Anderson et al. 2003).

A study at the Old Rifle site in Colorado identified an increase in the population of Gram-positive SRB known to reduce U(VI) during the sulfate reduction phase (Anderson et al. 2003). Another study at the same site showed a significant bloom of Firmicutes at one location (Vrionis et al. 2005). Finally, a third study showed that after Fe(III) and sulfate reduction, a new phase started during which sustained U(VI) removal was observed even after acetate amendments were stopped (N'Guessan et al. 2008). Firmicutes were identified as responsible for the removal of U(VI) during that phase and found to adsorb rather than reduce U(VI). The results of these three studies are consistent with a potential role of *Desulfotomaculum*-like bacteria in U(VI) immobilization after the completion of Fe(III) reduction and possibly sulfate reduction.

In summary, the results presented here show that U(VI) reduction by *D. reducens* is insensitive to the presence of nitrate and sulfate but is inhibited by iron and suggest that this microorganism and others with similar characteristics (e.g., spore forming *Clostridium* relatives) may play a role in the redox cycling of uranium in the subsurface after Fe(III) reduction has occurred.

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